Materials and methods

Materials and Methods are available in the online-only Data Supplement

Materials and animals

Allophycocyanin (APC)-labeled anti-mouse CD41 antibody (cat# 133913), and carboxyfluorescein succinimidyl ester (CFSE, cat# 423801) were purchased from Biolegend (San Diego, CA). APC anti-human CD41 antibody (clone HIP8, cat# 559777) was from BD Pharmingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-labeled anti-VWF (cat# P150-1) and FITC anti-mouse GPIbα antibodies (Xia.G5 and Xia.G7, cat# M040-1 and M042-1) were from Emfret Analytics (Eibelstadt, Germany). FITC-labeled SZ2 antibody (cat# IM0648U) was from Beckman Coulter (Indianapolis, IN), and Fura-2 AM (cat# 50033-1) is from Biotium (Fremont, CA). Fluorescein-labeled Erythrina Cristagalli Lectin (ECL, cat# FL-1141) was obtained from Vector Laboratories (Burlingame, CA). Apyrase (cat# A7646-500UN) and prostaglandin I_2 (PGI_2, cat# P6188-1MG) were from Sigma-Aldrich (Saint Louis, MO). Green fluorescent protein-fused lactadherin C2 domain (GFP-LactC2) and anti-MSD monoclonal antibody 5G6 were produced as described[1]. Gas-permeable bag (cat# PL07) was from OriGen (Austin, TX). OS1 peptide (CTERMALHNLC)[2] was synthesized by Genscript (Piscataway, NY) and purified to >95% by reverse-phase HPLC using a C4 column.

C57BL/6J (wild-type, WT) mice and $VWF^{-/-}$ mice were procured from Jackson Laboratory. Transgenic mice expressing only human GPIbα (hTg) has been described[3]. Six- to 8-week-old mice were used in the study as approved by the IACUC of Emory University. Since there has been no literature to suggest, and no reason to believe, that platelet GPIbα-VWF interaction and related GPIb-IX signaling are affected by sex, both male and female male mice were used in this study. All animals were randomized before the experiment, and investigators were blinded to group allocation during data collection. Human whole blood was drawn from healthy volunteers in 0.38% sodium citrate. The informed consent and related protocols were approved by Emory University Institutional Review Boards.

Refrigeration of platelets

Whole blood from WT, $VWF^{-/-}$ or hTg mice was collected into sodium citrate and platelet-rich plasma (PRP) isolated as described[4]. Prepared PRP was supplemented with 0.02 U/ml apyrase and 0.1 μg/ml PGI_2. For the mixed storage experiment, platelets and plasma were further separated by centrifugation. Platelets were washed once with Tyrode's buffer and divided into two equal parts. One part was gently resuspended in WT plasma and the other in $VWF^{-/-}$ plasma. The reconstituted PRP (2.5×10^5/μl platelets, ~1ml) was refrigerated in a gas-permeable bag at 4°C for 24 hours. Unless otherwise noted, after refrigeration PRP was rewarmed to 37 ˚C for 10 min before further characterization.

Measurement of VWF binding to platelets

Each PRP sample was treated with 5 µl FITC anti-VWF antibody (concentration not listed, used according to manufacturer's instruction), and 5 µg/ml APC anti-mouse CD41 (Biolegend) at RT for 10 min. The platelets were fixed with 4% PFA and analyzed on a Becton-Dickinson FACS Canto II instrument. The platelet population is gated by APC anti-mouse CD41. VWF binding was quantitated by mean fluorescence intensity (MFI) of the platelet population, subtracted by that of the negative control (sample treated with rat FITC anti-mouse IgG), and normalized with the relative MFI of fresh platelets (without storage) as 1.

Uniform shear treatment and measurement of platelet signaling

An aliquot of PRP sample (60 µl, containing 2.5×10^5/µl platelets) was uniformly sheared at 10 dyn/cm^2 for 5 min on a Brookfield Cap 2000+ cone-plate viscometer at RT largely as described[5]. In the case of no shear treatment, PRP was placed on the viscometer but no shear was applied. After shear treatment, platelets were collected and incubated immediately with 5 µM Fura-2 AM, 0.8 µM GFP-LactC2, 20 µg/ml ECL, 5 µl FITC anti-mouse GPIbα antibody (concentrated not listed, used according to manufacturer's instruction), 1:100 diluted FITC-labeled SZ2 (concentration not listed, used according to manufacturer's instruction), or 0.5 µg/ml FITC-labeled 5G6 for 10 min. In the case of 5G6 binding assay, 5G6 and 2mM EDTA were added before rewarming and shear treatment. After shear, the platelets were then fixed by 4% PFA and analyzed by flow cytometry[5]. The platelet population is gated by APC anti-mouse CD41. The signal intensities were quantitated by MFI of the platelet population, subtracted by that of the negative control, and normalized with the relative MFI of fresh platelets (without storage) as 1.

Post-transfusion recovery and survival of platelets in vivo

Fresh or refrigerated platelets in PRP were labeled with 2 µg/ml CFSE, and concentrated (750 g, 5 min) in the presence of apyrase and PGI_2[6]. CFSE-labeled platelets (100 µl) were infused via retro-orbital plexus into a WT mouse at 10^8 platelets per 10 g of body weight. At indicated time points afterwards, ~30 µl of whole blood was collected via the facial vein of infused mouse into heparinized capillary tubes (Fisher Scientific, West Chester, PA) and incubated with anti-mouse CD41 antibody for 30 min. All samples were then treated with 200 µl RBC Fix/Lyse solution (eBioscience, San Diego, CA) for 10 min, centrifuged at 750 g for 5 min, and platelet pellets were washed once, resuspended in 100 µl PBS, then fixed in 100 µl 4% PFA for flow cytometry analysis[6]. Platelet recovery was calculated as $\frac{CFSE^+\ cells}{anti{-}mCD41^+\ cells + CFSE^+\ cells}$, and normalized to that of fresh WT platelets at 1-hour post transfusion as 100%. Platelet survival (half-life time, $T_{1/2}$) was calculated using one phase exponential decay[7, 8].

Statistics
All the data were consistent with normal distribution and equal variance. The results are reported as mean ± SD. Differences between the groups were statistically analyzed by ANOVA with Bonferroni's post-hoc test or Student's t-test as specified in each figure. $P<0.05$ was considered as statistically significant.

Reference

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